Phorbol ester treatment impairs hormone- but not stable GTP analog-induced inhibition of adenylate cyclase

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Treatment of intact human platelets and S49 lymphoma cyc⁻ cells with the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate, impairs GTP-dependent and hormone-induced inhibition of adenylate cyclase, an action mediated by the inhibitory coupling protein N_i. In contrast, receptor-independent activation of N_i with subsequent adenylate cyclase inhibition induced by the stable GTP analog, guanosine 5′-[γ-thio]triphosphate, was affected in neither the potency nor onset of N_i activation by the stable GTP analog, in both membrane systems studied. The data indicate that modification of N_i following phorbol ester treatment does not impair its activation by stable GTP analogs.

Adenylate cyclase *Inhibitory coupling protein N_i* *Phorbol ester* *Protein kinase C* *Inhibitory hormone* *GTP analog*

1. INTRODUCTION

We have recently reported that treatment of intact human platelets and S49 lymphoma cyc⁻ cells with the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), impairs inhibition of adenylate cyclase by hormonal factors [1,2]. Furthermore, a similar effect with regard to impairment of GTP-dependent and hormone-induced inhibition of adenylate cyclase was observed when purified, Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) [3] was added to membranes of human platelets [4]. Finally, partially purified protein kinase C was shown to phosphorylate the guanine nucleotide-binding α-subunit of the inhibitory coupling protein N_i of the adenylate cyclase system [2]. This phosphorylation was observed with the purified N_i component and apparently also with the N_i protein present in intact and solubilized platelet membranes [2]. These data suggest that phosphorylation of N_i by protein kinase C impairs its function in the inhibitory hormone signal transduction to the adenylate cyclase.

In addition to being activated by agonist-stimulated hormone receptors in the presence of GTP, the N_i component can be directly activated by stable GTP analogs such as guanosine 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[β,γ-imido]triphosphate even without hormones present. Such an activation of N_i has been observed in various membrane systems, including those of human platelets and S49 lymphoma cyc⁻ cells [5–7]. Activation of N_i with subsequent inhibition of adenylate cyclase by stable GTP analogs is preceded by a considerable lag phase, which is not seen with inhibitory hormones and GTP [5–7]. Therefore, we investigated whether treatment of intact human platelets and S49 lymphoma cyc⁻ cells with TPA may also affect the receptor-independent activation of N_i by stable GTP analogs. We report here that, in contrast to hormone-induced inhibition of adenylate cyclase, inhibition of adenylate cyclase by the stable GTP analog GTP[S] is not impaired by the phorbol ester treatment.

2. MATERIALS AND METHODS

GTP[S] was obtained from Boehringer Mannheim. The other materials used were from previously described sources [1,2,4]. Pretreatment of intact human platelets and S49 lymphoma cyc⁻

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cells with TPA at 1 μM was carried out as in [1,2]. Preparation of membranes from control and TPA-pretreated cells was performed as in [5,7].

Adenylate cyclase activity of human platelet membranes (3–5 μg protein/tube) was determined with a reaction mixture containing 50 μM [α-^{32}P]-ATP (0.2–0.3 μCi/tube), 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM cyclic AMP, 1 mM dithiothreitol, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 2 mg/ml bovine serum albumin, 20 μM forskolin and the additions indicated in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 μl. For measurement of adenylate cyclase activity in membranes of S49 lymphoma cyc$^-$ cells (15–25 μg protein/tube), the reaction mixture was the same as that above with the exceptions that $MgCl_2$ was replaced by 200 μM $MnCl_2$ and EGTA was omitted. Furthermore, the concentration of forskolin was 100 μM. The membranes were preincubated with the complete reaction mixture including forskolin and the GTP analog for 10 min at 25°C to overcome the lag phase of GTP[S]. Thereafter, measurement of adenylate cyclase activity was started by addition of labelled ATP and conducted for 10 min at 25°C. In time course experiments, the membranes were preincubated for 7 min at 25°C with the reaction mixture including forskolin but without GTP[S] present. This preincubation was necessary in order to have linear rates of cyclic AMP formation, since the stimulatory effect of forskolin, the presence of which was required to observe the inhibition by GTP[S], is also preceded by a short lag period [5]. Measurement of cyclic AMP accumulation was initiated by the addition of [α-^{32}P]ATP without and with GTP[S] and continued for various time periods at 25°C. Stopping of the reaction and isolation of cyclic AMP formed were as in [8]. The assays were performed in triplicate with an intra-assay variation of less than 5% of the means and were repeated twice with results comparable to those shown herein.

3. RESULTS

As shown before [1], treatment of human platelets with the phorbol ester TPA (1 μM) resulted in a large reduction in adenylate cyclase inhibition caused by the inhibitory hormone epinephrine, as measured in membrane preparations in the presence of 10 μM GTP (fig.1). In control membranes, epinephrine reduced enzyme activity by 40–50%. In membranes of platelets pretreated with TPA, epinephrine-induced adenylate cyclase inhibition was only observed at high agonist concentrations with a marked reduction in the maximal extent of inhibition. In contrast, inhibition of the forskolin-stimulated platelet adenylate cyclase by the stable GTP analog GTP[S] appeared not to be affected by the phorbol ester treatment. Both the maximal extent of inhibition and the potency of GTP[S] were very similar in control membranes and membranes of human platelets pretreated with TPA.

The stable GTP analog-induced inhibition of adenylate cyclase is preceded by a considerable lag phase, the duration of which is inversely related to the GTP[S] concentration [5] and which appears to reflect the time required to activate N_i fully. Therefore, we studied whether the TPA treatment may affect this turn-on reaction of N_i by GTP[S]. As

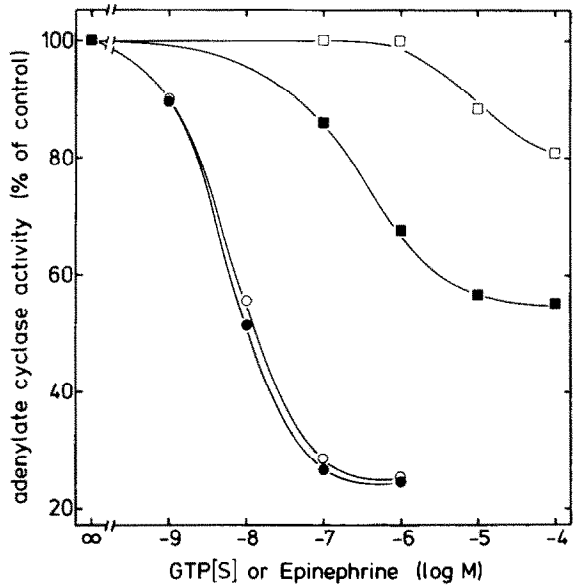

Fig.1. Influence of TPA treatment of intact human platelets on hormone- and GTP[S]-induced inhibition of adenylate cyclase. In membranes of control platelets (closed symbols) and platelets treated with 1 μM TPA (open symbols), adenylate cyclase activity was determined as described in section 2 at the indicated concentrations of epinephrine (■,□) or GTP[S] (●,○). For study of the effect of epinephrine, GTP (10 μM) was included in the reaction mixture. Adenylate cyclase activity is given as percent of control activities measured in the absence of epinephrine and GTP[S].

shown in fig.2, in control membranes inhibition of cyclic AMP formation by the adenylate cyclase caused by the addition of GTP[S] (30 nM) was preceded by a small but significant lag phase of about 2.5 min at 25°C. The maximal extent of inhibition achieved was 65–70%. In membranes pretreated with TPA (1 μM), control forskolin-stimulated activity was increased somewhat, by about 15%, as reported in [1]. As in control membranes, the inhibition of cyclic AMP formation in membranes of TPA-pretreated platelets induced by GTP[S] was preceded by a lag phase, the duration of which (2.1 min) was similar to that observed in control membranes. Furthermore, the maximal extent of inhibition caused by GTP[S] was identical in control membranes and membranes of TPA-pretreated platelets.

In membranes of S49 lymphoma cyc$^-$ variants, which lack the guanine nucleotide-binding α-subunit of the stimulatory coupling component N_s [9], stable GTP analogs apparently only interact with the N_i component causing adenylate cyclase inhibition [6,7]. It has been shown that, similarly to human platelets, treatment of intact S49 lymphoma cyc$^-$ cells with TPA impairs GTP-dependent inhibition of adenylate cyclase by the inhibitory hormone, somatostatin, whereas activation of adenylate cyclase by forskolin is apparently not affected [2]. Therefore, we also determined whether the receptor-independent activation of N_i by GTP[S] with subsequent inhibition of adenylate cyclase is affected by the phorbol ester treatment. As shown in fig.3, in membranes of control S49 lymphoma cyc$^-$ cells, GTP[S] reduced adenylate cyclase activity by maximally 40%, with an IC_{50} of about 10 nM. In membranes of S49 lymphoma cyc$^-$ cells pretreated with TPA (1 μM), a treatment which impaired somatostatin-induced inhibition [2], both the potency and efficiency of GTP[S] in causing inhibition of adenylate cyclase were identical to those in control membranes.

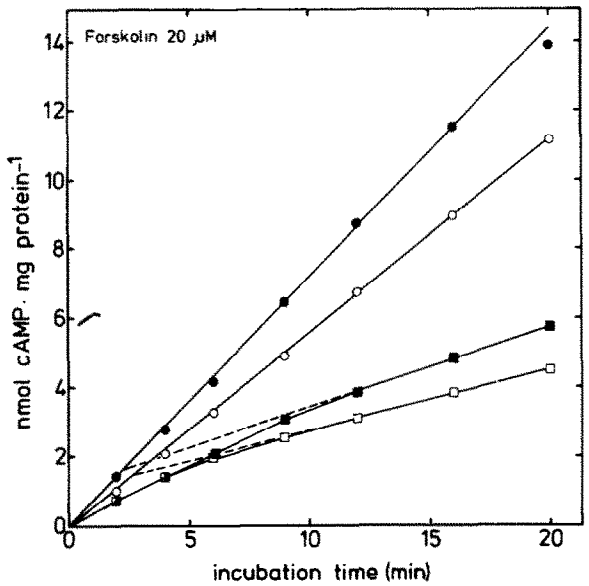

Fig.2. Time course of GTP[S]-induced inhibition of adenylate cyclase in membranes of control and TPA-pretreated platelets. Cyclic AMP accumulation was studied in membranes of control (open symbols) and TPA-treated platelets (closed symbols) in the absence (○,●) and presence of 30 nM GTP[S] (□,■) for the indicated periods of time. GTP[S] was added to the reaction at zero time. The intercepts of the control curves (○,●) with the dashed lines indicate the extrapolated time lags of GTP[S] action.

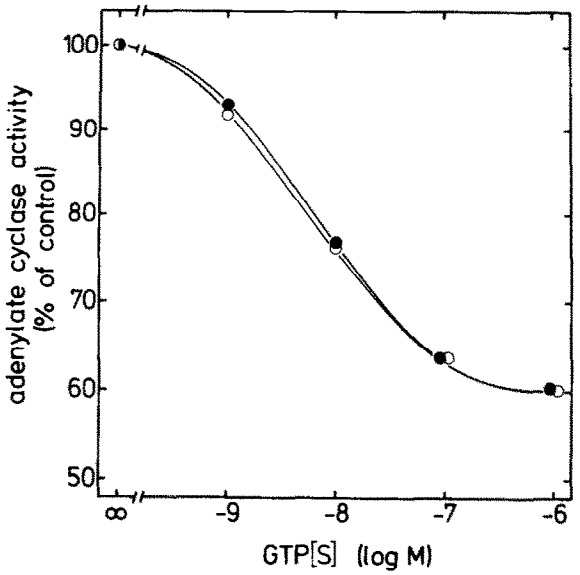

Fig.3. Influence of TPA treatment of intact S49 lymphoma cyc$^-$ cells on GTP[S]-induced inhibition of adenylate cyclase. Adenylate cyclase activity was determined in membranes of control (○) and TPA (1 μM)-pretreated (●) S49 lymphoma cyc$^-$ cells at the indicated concentrations of GTP[S] as described in section 2.

4. DISCUSSION

Treatment of intact human platelets and S49 lymphoma cyc$^-$ cells with the phorbol ester TPA inhibits GTP-dependent and hormone-induced in-

hibition of adenylate cyclase, a reaction mediated by the inhibitory coupling protein N_i [1,2]. This impairment seems to be due to phosphorylation of the guanine nucleotide-binding α-subunit of N_i by TPA-activated protein kinase C [2]. In this regard, the action of TPA appears to be similar to that of pertussis toxin, causing ADP-ribosylation of the α-subunit of N_i [10,11] with subsequent impairment of the inhibitory hormone signal transduction to the adenylate cyclase [12]. Furthermore, as in the case shown here for membranes of TPA-treated cells, treatment with pertussis toxin does not abolish N_i-mediated inhibition of adenylate cyclase by stable GTP analogs [13–15]. However, treatment with pertussis toxin causes a large increase in the lag phase of GTP[S] inhibitory action, suggesting that the activation of N_i is impaired by its ADP-ribosylation [16]. In contrast, as shown here for membranes of TPA-treated platelets, the phorbol ester treatment did not cause an increase in the lag phase of the inhibitory action of GTP[S]. These data suggest that, in contrast to pertussis toxin-ADP-ribosylated N_i, phosphorylation of N_i by protein kinase C does not impair the activation process of N_i following binding of stable GTP analogs. In this regard, it is worth bearing in mind that both ADP-ribosylation and phosphorylation of N_i by pertussis toxin and protein kinase C, respectively, are inhibited by pretreatment with GTP[S] [2,17]. However, whereas ADP-ribosylation of α_i depends on the presence of the $\beta\gamma$-subunit complex, phosphorylation of α_i by protein kinase C is inhibited by this complex [2,18].

If, as suggested by the present data, the activation reaction of N_i is not inhibited following phosphorylation by protein kinase C, it may be speculated that the inactivation reaction of N_i apparently involving hydrolysis of α_i-bound GTP to GDP and association of α- and $\beta\gamma$-subunits is increased. Such an increased hydrolysis of GTP would not affect the action of stable GTP analogs. It is also feasible that N_i phosphorylated by protein kinase C hydrolyzes GTP to GDP before the component can isomerize to the active state. Since GTP is required for the active state, this state will not be formed in an amount sufficient to induce inhibition of adenylate cyclase. Such a premature hydrolysis of GTP would also not affect the activation reaction of N_i by stable GTP analogs. Finally, is has not been proven that the mechanism of N_i activation by stable GTP analogs such as GTP[S] is identical to that caused by GTP and agonist-activated hormone receptors.

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